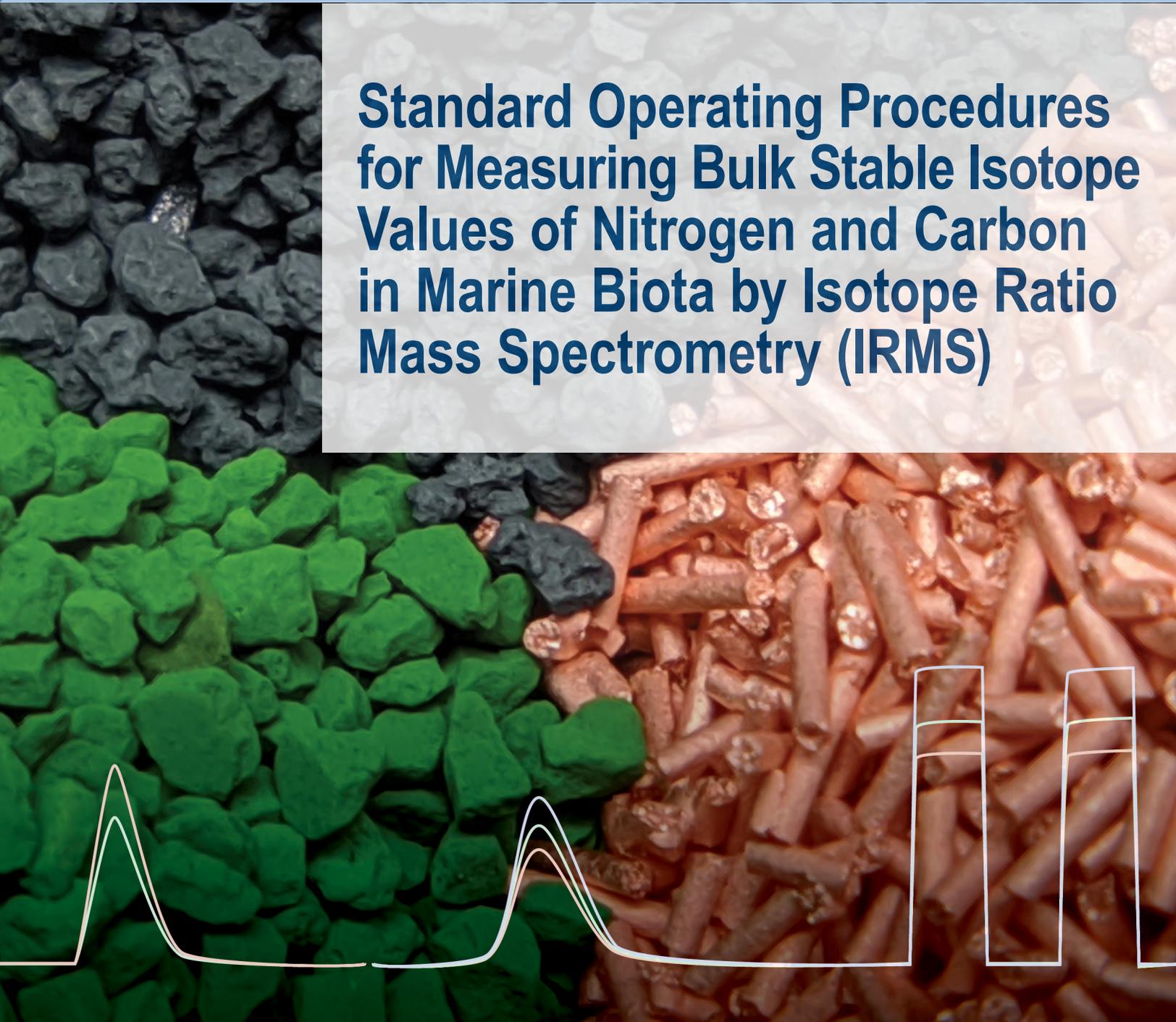




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# Standard Operating Procedures for Measuring Bulk Stable Isotope Values of Nitrogen and Carbon in Marine Biota by Isotope Ratio Mass Spectrometry (IRMS)



**February 2020**

U.S. DEPARTMENT OF COMMERCE

National Oceanic and Atmospheric Administration  
National Marine Fisheries Service  
Northwest Fisheries Science Center

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Cover image: Reactor reagents used for bulk carbon and nitrogen analysis. Clockwise from the top: silvered cobaltous, reduced copper, chromium oxide. Also depicted is an outline of nitrogen and carbon dioxide peaks; from left to right: N<sub>2</sub> sample peak, CO<sub>2</sub> sample peak, and two CO<sub>2</sub> reference gas peaks. Photograph by J. Gates, NMFS/NWFSC.

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**U.S. DEPARTMENT OF COMMERCE**

National Oceanic and Atmospheric Administration  
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Northwest Fisheries Science Center

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## 1.0 Introduction

The isotope ratio mass spectrometer (IRMS) housed at the Northwest Fisheries Science Center (NWFSC) is used to conduct accurate and precise determinations of isotope ratios of carbon and nitrogen. NWFSC's Environmental Chemistry Program lab facility includes a Thermo Fisher Scientific's Flash 2000 Elemental Analyzer coupled with the Conflo IV interface and the Delta V Advantage IRMS. These systems are complex and sensitive and require detailed care and regular maintenance to ensure a high level of performance. This processed report is written to be used in conjunction with hands-on training. It is meant to be treated as a living document and will be updated as changes are made in the procedures.



This report provides step-by-step instructions for sample preparation, instrument operation, maintenance of the instruments, sample archival, quality assurance (QA) and quality control (QC) measures and criteria, and data reporting. The *Quality Assurance Plan for Analyses of Environmental Samples* (Sloan et al. 2019) was referenced throughout Section 13. Reference information can be found at the end of this report.

## 1.1 Scope and Applicability

This method was developed using approximately 1 gram of marine biota tissue and produces a homogenized, stable sample that can be stored at room temperature indefinitely. This homogenate can be sampled multiple times for bulk carbon and nitrogen stable isotope analysis as well as compound-specific  $^{15}\text{N}$  analysis of amino acids.

It takes approximately 3–7 days to process a sample set submitted for bulk stable isotope analysis, depending on sample set size and whether or not the set has to be lipid extracted. It takes about 8 minutes per sample to run on the EA-IRMS for bulk carbon and nitrogen stable isotope ratios.

## 1.2 Summary of Procedure

1. Tissue samples are subsampled and placed in a labeled scintillation vial with cap. Samples are placed in a  $-80^{\circ}\text{C}$  freezer for at least an hour or until they are frozen solid, and then placed in a freeze dryer for 24 hours to remove water.
2. If lipid removal is required, then samples are loaded onto an accelerated solvent extractor (ASE) 350 to obtain lipid-free samples. For more in-depth details of the ASE 350 extraction method, see Herman et al. 2005.
3. Samples that do not require lipid removal or post-lipid removal samples, are homogenized using a Mixer Mill (SPEX SamplePrep 5100).
4. Homogenized samples and standards are then weighed into individual tin capsules, and these are run on the IRMS.
5. Data from the IRMS are run through a standard QA/QC and data processing procedure, and finalized data are reported.

## 1.3 Definitions

**ASE:** accelerated solvent extractor

**CSIA:** compound-specific isotope analysis

**DQO:** data quality objective

**EA:** elemental analyzer

**IRM:** internal reference material

**IRMS:** isotope ratio mass spectrometer

**SOP:** standard operating procedure

**SRM:** standard reference material

## 1.4 Safety

This method uses numerous chemicals and reagents that are considered hazardous for flammability and toxicity. Therefore, a fume hood is used when working with hazardous materials to minimize exposure of the analyst to solvent vapors. When working with all chemicals, a minimum of nitrile gloves and eye protection are worn. Additional laboratory safety practices are followed according to the NWFSC *Chemical Hygiene Plan* and the NWFSC *Chemical Waste Management Guide*. The Manage Material Safety Data Sheets (MSDS) should be reviewed for all chemicals and reagents prior to starting this procedure.

## 2.0 Documentation and Recordkeeping

It is imperative that all steps throughout the process procedure (e.g., sample preparation, maintenance of the instrumentation, and operation of the instrumentation) are recorded in the appropriate logbooks with corresponding sample set number, dates, and sample preparer/instrument operator initials. It is also important to update the status of the sample set in Environmental Chemistry's Google Drive under *SI Current Set Status*.

## 3.0 Equipment List

It is crucial that all scalpels, spatulas, forceps, pulverization chambers, and steel bearings are cleaned with oil-removing detergent (Micro-90 detergent), an appropriate scrub brush, ample water rinses, followed by sonication in an appropriate solvent. These steps will help prevent potential contamination that could affect the isotope measurements.

### 3.1 Materials, Per Sample

#### Non-Lipid Extraction Samples

- 1 ea. 20-mL scintillation vial with polyethylene lined cap
- 1 ea. clean stainless-steel pulverization chamber with bearing
- 1 ea. 4×6 tin capsule

#### Lipid Extraction Samples

- 2 ea. 20-mL scintillation vial with polyethylene lined cap
- 5.5-cm round glass-fiber filter — VWR 696
- 250-mL Accelerated Solvent Extractor (ASE) collection bottle
- 3 glass-fiber filters per ASE cell, fired overnight at 400°C in a muffle furnace
- 1 clean ASE cell per sample (see [Section 6.5](#))
- 1 ea. 4×6 tin capsule

### 3.2 Solvents and Cleansers

- methylene chloride (for ASE)
- acetone (for cleaning supplies via sonication)
- Micro-90 detergent

### 3.3 Other Materials and Apparatus

- Accelerated Solvent Extractor ASE 350 — Dionex Corp ASE
- desiccator
- Virtis Freezemobile 12XL
- Mixer Mill — SPEX SamplePrep 5100
- ultrasonic cleaner — Branson 5800
- stainless steel scalpel with blades
- stainless steel spatulas
- forceps
- paper clips, rinsed with acetone
- 96-well trays
- six-place balance — Mettler Toledo Excellence Plus
- two-place balance — Adventurer Ohaus AR3130
- -20°C freezer for temporary sample storage
- -80°C freezer for long-term sample storage and pre-freeze dry
- pilling tools
- frozen ice block
- Delta V Advantage IRMS
- Thermo Scientific Flash 2000 Organic Elemental Analyzer Flash HT Plus
- Thermo Scientific Conflo IV

## 4.0 Sample Preparation/Dissection

Prior to sample preparation, refer to the Instructions for Submission of Samples for Stable Isotopes Analyses ([Appendix A](#)) as well as the Submission Form for Stable Isotopes Analyses ([Appendix B](#)). Once the sample set has been scheduled, it is best to locate the samples and ensure they are ready to be subsampled. If the samples need to be homogenized, do so. Make sure to keep all paperwork that is associated with the sample set together in the corresponding folder.

### 4.1 Labeling Sample Vials

Begin by preparing cloth labels for the entire set by writing the assigned lab number, field ID, and the set number on the label for each sample (set number is found on white sheet). Attach each label to a new glass scintillation vial and record the lab number on the vial lid using a felt pen. If the set requires lipid extraction, the initial scintillation vial only requires the lab number written with a felt pen on the cap and vial. Post-lipid extraction, the sample will be transferred to a new scintillation vial labeled with cloth labels that include the lab number, field ID, and the set number on both the cap and vial.

## 4.2 Preparing Dissection Equipment (if applicable)

Cut aluminum foil squares for each sample. In a fume hood, rinse the foil surface with acetone and let it dry. Pull the frozen ice block from the freezer and place in the hood. Cover the working surface of the block with new acetone-rinsed aluminum foil. After assembling the blades onto the scalpel handles, acetone-rinse the blades in a fume hood and set them on a clean piece of aluminum foil. Place a QuickSmart automatic scalpel blade remover next to your dissection surface. Locate clean forceps and place them near your work space.

## 4.3 Dissecting Tissue from a Single Specimen

Remove the tissue from the sample container using forceps and place it on the clean aluminum foil atop of the ice block. For the skin analysis, carefully cut the skin from the field sample using a clean scalpel, being careful to remove all of the blubber and muscle. For blubber analysis, carefully cut the blubber from the field sample using a scalpel, being careful to remove all of the skin. For all other tissues, remove a small piece from the middle section, to avoid the areas of the sample that might have been exposed to freezer frost. Cut the sample into small pieces (~0.1 g or smaller) using the scalpel and place them separately in the labeled scintillation vial, placing them back in the freezer between each sample.

## 4.4 Subsampling from a Homogenized Sample or Sample Composite

Place all of the sample jars out to thaw for at least 45 minutes prior to subsampling. After the samples have thawed, scoop out a small amount of the sample using a clean spatula, trying to avoid the very top layer. Use a new, clean spatula for each sample. Place the labeled 20-mL scintillation vial on the two-place balance and weigh out a small amount of the sample. The weights do not need to be recorded and are only used to make sure enough sample has been subsampled and that sample material is not wasted. Approximate subsample masses are listed below:

- For whole body fish samples, it is best to weigh out at least 0.5 g of tissue, keeping in mind that there might be pieces of skin or bone in the subsample that cannot be ground to a powder.
- For fish muscle samples, subsample 0.2–0.4 g of tissue.
- For mussel or shellfish samples, weigh out at least a 1-g sample due to the increased amount of water in each sample compared to blubber or fish muscle.

## 4.5 Cleaning the Dirty Spatulas

After completing the subsampling step, take the dirty spatulas to the sink and scrub off any residual tissue using Micro-90 soap. It is best to work with gloves, as the detergent is concentrated and corrosive. Once the spatulas are clean of tissue, place them in the perforated stainless steel insert inside of the sonicator and fill with water and Micro-90 detergent to the fill line. Never operate the sonicator without an insert.



After the sonication step is completed, drain the water from the unit using the valve located on the left side. Open the valve and let the water flow down the drain located in the fume hood. Remove the insert and rinse everything under water. Lay the spatulas out on absorbent paper next to the sink to dry overnight.

When the spatulas have completely dried, place them in the solid sonicator tray insert and submerge with acetone in a fume hood. Make sure to use the appropriate nitrile gloves while working with the acetone. Fill the sonicator with 1,650 mL of water before inserting the solid tray insert into the unit. Set the unit to sonicate for 30 minutes.

After the sonication step is completed, drain the water from the unit using the valve located on the left side of the unit. Open the valve and let the water flow down the drain located in the fume hood. Wearing nitrile gloves, remove the solid tray insert and drain the acetone into a waste bucket. Pour the acetone waste in the waste container labeled **100% acetone**, located in the fume hood. Leave the insert to sit in the fume hood until all of the solvent has completely evaporated (at least 30 minutes). Return the clean spatulas from the insert, using gloves, to the glass jar labeled **Spatulas**.

## 5.0 Freeze Drying

After all of the samples have been subsampled, loosen all of the caps of the samples and place them in a  $-80^{\circ}\text{C}$  freezer for at least an hour prior to freeze drying.



## 5.1 Preparing the Freeze Dryer

Begin by checking the level of the oil in the rough pump to make sure that there were no leaks between runs. The rough pump can be accessed by opening the door located to the right of the condensing unit. Check the level of the oil in the sight glass and act accordingly. If the oil level is OK to operate, latch the refrigerator door all the way shut (it should be open from the last run so that the chamber can dry out). Using the **Refrigerate** switch, turn the refrigerator on and let it cool for the hour that your samples are in the  $-80^{\circ}\text{C}$  freezer (until the freeze dryer reaches at least  $-30^{\circ}\text{C}$ ).

## 5.2 Loading the Samples into the Freeze Dryer Chamber

Remove the lid to the chamber and place it on its side on the table. Remove the tray from inside of the chamber so that the samples can be placed on the shelves easily. Place the sample vials on the freeze dryer trays and be sure the caps have been slightly loosened so that moisture can escape. Only place samples on the middle and bottom shelves (not on top of the rack). During the vacuum change, samples may come out of their vials and contaminate nearby samples, so it is advisable to keep the caps on but loosened.

Next, place the vacuum chamber lid on, making sure it is centered around the lip of the chamber, and close the white valve located on the top of the lid. The valve is closed when the handle is in the back and the small dimple is visible in the valve window. Switch on the vacuum using the switch labeled **Vacuum**. Once the freeze dryer is sealed, start the vacuum pump. After about 15 minutes it is best to check back and make sure that the vacuum sensor is reading a low vacuum (approximately 15 Millitorr). For sufficient sample lyophilization (water removal), let the unit run for approximately 24 hours.



Open.



Closed.

Record the sample set information, the date, your initials, the matrix as well as the number of samples in the freeze dryer in the freeze dryer logbook. Be sure to update the file entitled *SI Current Set Status*, which is found in Environmental Chemistry's Google Drive.

## 5.3 Removing the Samples from the Freeze Dryer

When the samples are dry, stop the vacuum pump by switching the **Vacuum** switch off. Switch off **Refrigerate** as well. Slowly open the white valve located atop the vacuum chamber lid and let the air out—slowly, so that the air surge does not disrupt the samples. Once there is no longer vacuum, remove the chamber lid, setting it on its side, and remove the tray from inside the chamber. Remove the samples, tighten the caps, and place them in order in the vial tray.

Leave the condenser door cracked open, with a few paper towels in the chamber to help speed up the drying process. Examine the O-rings located on the condenser door and vacuum chamber for any tears or debris, and make note if they need to be lubricated or replaced. Place the vacuum chamber lid back on top of the unit so that dust and debris do not collect in the unit while idle.

**If the samples do not require lipid extraction, skip Sections 6 and 7. Proceed to Section 8.**

## 6.0 Lipid Extraction on the Automated Solvent Extractor (ASE)

The ASE cells that are used to lipid-extract samples to be run for stable isotope analysis are kept separate from the others (they are only sonicated in Acetone between runs).

### 6.1 Prepare ASE Cells

Set out all of the clean ASE cells that you plan to use and, using a felt pen, label the body of each cell with the sample number. Unscrew the top cap of the cell and, using forceps, place two fired glass-fiber filters inside. Using the appropriate black rod, push the filters down to the bottom of the cell. Make sure that the bottom cap is on tight.

### 6.2 Prepare Samples

Wearing gloves, take a 5.5-cm diameter glass-fiber filter, fold it in half twice, and open one side to make a filter cone. Be careful when handling the glass-fiber filters; they are fragile. Clip three of the folded sides of the filter cone together with a clean paper clip and attach the clip to the edge of a clean, labeled ASE cell in order to hold the cone open.

Using a clean spatula, scoop the sample out of the vial and place it in the filter cone. Because the samples are dry, you can use one spatula for the whole set, making sure to wipe it off between samples with a paper towel. Wearing gloves, fold the cone in half and paper clip the cone closed. Place the sample pocket inside of the appropriate labeled cell. Using forceps, put one glass-fiber filter on top of the sample and push it down gently with the black rod. Place the cell cap on tightly and load the sample on the ASE. Repeat this process until all of the cells have been used.

If there is too much sample to fit into one filter cone, another cone should be made or the rest of the sample should be discarded. The vial that the sample was freeze-dried in will be discarded in the **Broken Glass** box after the sample has been loaded in the ASE cell.



Most SI sets contain too many samples to lipid-extract in a single run on the ASE, so they will require multiple runs on the ASE and proper cleaning between sample sets. See [Section 6.5](#) for how to clean the ASE cells.

### 6.3 Loading/Operating the ASE

Begin by opening the valve on the nitrogen tank so that the system has gas flow. Load all of the cells on the top carousel of the ASE in order of sample number. Make sure there is a waste bottle/vial for each cell that has been loaded as well as a rinse bottle/vial in tray positions R1 and R2. Remove the solvent reservoir, placing the sparge in a clean ASE vial temporarily, and cap the bottle. Working in the hood with the appropriate nitrile gloves, fill the solvent reservoir with methylene chloride. Hook the solvent reservoir back up to the instrument.

Using the keypad on the instrument, locate **Method 3**, the method used for lipid extraction. The parameters should match:

- Cycles: 2
- Purge: 60 seconds
- Static: 5 minutes
- Heat: 0 minutes
- Rinse Volume: 80%
- Temperature: 0°C
- Cell Type: SST
- Solvent Saver: Off
- Solvent A:1 Methylene Chloride

Double-check that you have the same number of cells as waste bottles, as well as rinse bottles in tray positions R1 and R2. Press the **Rinse** button to run a manual rinse, and repeat that step two more times. After the system has been thoroughly rinsed, press the **Start** button and allow the system to lipid-extract the sample set.

### 6.4 Unloading Samples from the ASE Post-Lipid Extraction

After all of the samples have been lipid-extracted, press the **Tray** button until the green indicator light moves to the left side of the button which releases the sample trays. Working with nitrile gloves, remove all of the waste bottles and pour the methylene chloride into the labeled methylene chloride waste bottle stored in a fume hood. Toss the perforated caps away and completely dry the bottles in the fume hood for several hours before recapping them for the next set. The bottles are reused for lipid extracting stable isotope samples or rinsing cells. As noted previously, it may take more than one round of ASE extractions to lipid-extract an entire SI sample set.

Remove the ASE cells from the upper tray and place them in a fume hood. Remove the cell cap and, using forceps, remove the sample from the cell and place it in a newly labeled scintillation vial. Loosely place the cap on the scintillation vial so that the solvent can

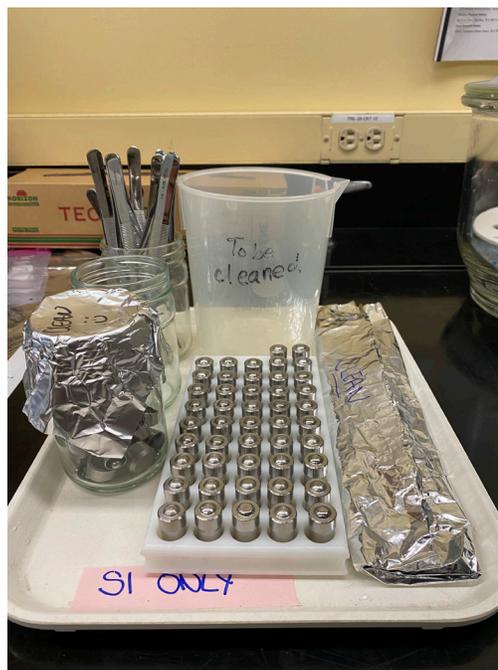
fully evaporate from the sample. Repeat these steps until all samples have been removed from the cells. Once the solvent has evaporated from the samples, tighten the caps and remove them from under the fume hood. Leave the filters in the hood until the solvent has evaporated from them and then dispose of them.

## 6.5 Cleaning the ASE Cells

After the ASE cells have been dismantled, place all of the caps in a cylindrical steel sonicator tub. Working with nitrile gloves, remove the sample numbers from the cell bodies with acetone and a paper towel and place the bodies in another cylindrical steel sonicator tub. Fill both tubs with acetone until all of the caps and bodies are submerged. Place the tubs inside the perforated sonicator insert tray and fill the tray around the tubs with water to the fill line. Sonicate the ASE cell parts for 30 minutes. After the sonicator has finished, pour the acetone into the appropriate waste container and transfer it to the flammable waste container under the fume hood. Lay all of the cell parts out on a clean piece of tin foil to dry in the fume hood. Once the solvent has fully evaporated from the cell parts, reassemble the cells and store them in their assigned space.

## 7.0 Homogenization

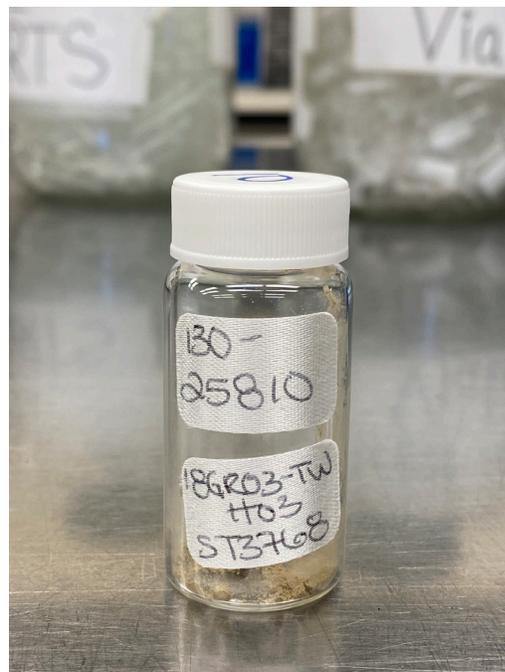
In order to achieve sample homogeneity, samples must be run through a Mixer Mill and ground for approximately three minutes using a steel ball bearing in a steel capsule.



## 7.1 Homogenizing Samples Post-Lipid Extraction

Begin by carefully opening the bundle with clean forceps or clean gloves. Unfold the filter cone and, using a clean spatula, transfer the dried sample into a clean pulverization capsule equipped with a steel ball bearing. Place the lid onto the capsule body, making sure that it is equipped with an O-ring. Clamp the chamber into the Mixer Mill, making sure that there is rubber cushioning on either side of the capsule inside of the clamp. Set the timer for 3 minutes and push the white button located in the middle of the timer dial to pulverize the sample.

When the Mixer Mill has finished, remove the chamber from the Mixer Mill and remove the lid from the body of the capsule. If the lid has become stuck to the body, lightly tap the capsule on its sides and try removing the lid again. Repeat as many times as it takes to remove the lid. Using a clean spatula, transfer the pulverized sample from the capsule and lid back into the labeled scintillation vial. Repeat this process for each sample.



Some sample sets may be larger than the number of Mixer Mill capsules we currently have, so be sure to properly clean the capsules between homogenization events. See [Section 7.3](#).

## 7.2 Homogenizing Samples without Lipid Extraction

Begin by carefully transferring the dried sample into a clean pulverization capsule equipped with a steel ball bearing. Place the lid onto the capsule body, making sure that it is equipped with an O-ring. Clamp the chamber into the Mixer Mill, making sure that there is rubber cushioning on either side of the capsule inside of the clamp. Set the timer for 3 minutes and push the white button located in the middle of the timer dial to pulverize the sample.

When the Mixer Mill has finished, remove the chamber from the Mixer Mill and remove the lid from the body of the capsule. If the lid has become stuck to the body, lightly tap the capsule on its sides and try removing the lid again. Repeat as many times as it takes to remove the lid. Using a clean spatula, transfer the pulverized sample from the capsule and lid back into the labeled scintillation vial. Repeat this process for each sample.

Some sample sets may be larger than the number of Mixer Mill capsules we currently have, so be sure to properly clean the capsules between homogenization events. See [Section 7.3](#).



### 7.3 Cleaning the Capsules, Spatulas, and Forceps

Take all of the dirty capsules, spatulas, forceps, and paperclips in the **To Be Cleaned** container to the sink in Room 253E. Fill the container with water and Micro-90 soap. Do not leave them to soak overnight, as the paper clips will start to corrode.

Wearing gloves, remove the spatulas and wipe them thoroughly with a soapy glove or bottle brush, getting any leftover material off before placing them in the solid stainless steel sonicator insert filled with Micro-90 soap and water.

Remove all of the paper clips from the container, thoroughly rinse them with water, and place them on the absorbent material by the sink to fully dry.

Remove the capsule bodies, rinse thoroughly with water, and, using a small scrub brush coated in Micro-90 soap, scrub out the bodies. Thoroughly rinse the bodies with water and place them in the same stainless steel sonicator insert.

Using a pair of forceps, remove all of the O-rings from inside the capsule lids, rinse thoroughly with water, and, using a small scrub brush coated in Micro-90 soap, thoroughly scrub out the lids. Rinse the lids with water and place everything in the stainless steel sonicator insert.

The steel ball bearings and O-rings left at the bottom of the **To Be Cleaned** container should be thoroughly rinsed with water until they are free of any biological material. Add them to the stainless steel sonicator insert and make sure that the soapy water covers everything in the tub. Add 1,650 mL of water into the sonicator before placing the solid stainless steel insert with all of the parts and spatulas into the sonicator. Sonicate for 30 minutes. When the sonicator is finished, remove the insert and rinse well with water, making sure that nothing is lost down the sink. Lay everything out on the absorbent paper next to the sink to dry overnight. Do not leave the utensils to sit overnight in the soapy water as the soap is corrosive.

After all of the tools have fully dried, place them back in the solid stainless steel insert and fill it with acetone in a fume hood, making sure everything is submerged (do not sonicate the paper clips with the tools). Make sure to wear appropriate gloves while working with solvent. Place the tub back in the sonicator after adding 1,650 mL of water to the unit, and run for another 30 minutes. When the sonicator is finished, wearing gloves, dump the solvent waste into a waste container in the fume hood and dispose of it in the appropriate flammable waste jug. Lay all of the tools out on a clean piece of foil under the fume hood to allow the solvent to fully evaporate (approximately 2 hours). When everything is dry, rebuild the capsules, placing a ball bearing in each one, and place the clean spatulas back with the rest on the tray.

After the paper clips have fully dried, place them in a clean beaker in the fume hood. Using an acetone squirt bottle, triple rinse the paper clips, draining them between each rinse. Let them sit under the fume hood until all of the solvent has evaporated from them. Once they have dried, place them back with the rest of the paper clips.

## 8.0 Weighing Samples

Throughout this final process, attention to detail is key. It is important to remember that the oils from your hands can contaminate samples, so it is best to always use clean tools and wear gloves. It is also imperative to double-check sample weights when inputting them into the template, as an error will throw off the calculation of the sample.

### 8.1 Building a Loading Sheet

The loading templates are found on the stable isotope server. Select the appropriate template and save it using the name of the set—for example, *ST1111 Loading*. Do not overwrite the template. Place the new loading file into the set folder found on the server under *Stable Isotope Projects*. Input the data found in the corresponding `setup.csv` file into the right corner of the loading template (starting with cell I25). The information will autofill in column Z and throughout the loading sheet. Type the set name into the box at the top of the page (cell I3), and fill out the information in cells D52–D56; the information will autofill on the next page.

Depending on how many samples have been assigned to the set, rows will have to be removed. Highlight the empty rows that are not needed and delete them, shifting the cells up. If the set is small, it is not necessary to run all of the standards. All sample sets must have five SRMs (two run at the beginning of the run, one run in the middle, and two run at the end of the sequence), and a minimum of 5 each of aspartic acid and histidine standards (two each at the beginning of the run, one each every 15 or fewer samples, and two each at the end of every run).

Once the loading sheet is fully filled out, print off a hardcopy to fill out the weights of your samples. The *Sample & Standard Weight Guide*, found on the third tab in the loading template, can be referenced if you are unsure of the weights for the standards and samples in the set.

### 8.2 Pilling of Samples for Analysis

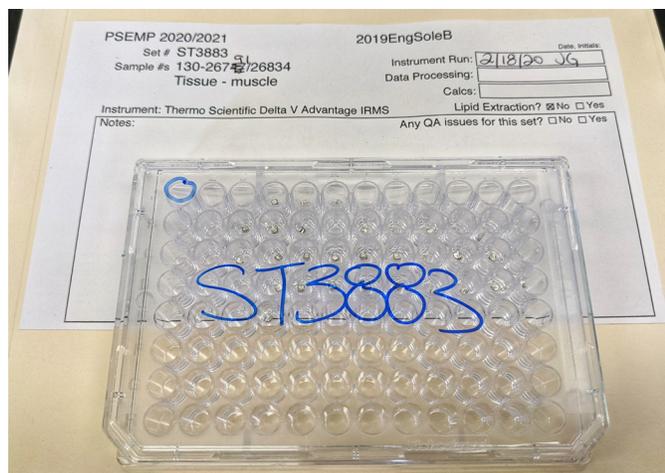
For all pilling operations, be extremely careful not to touch the tin cups, the working ends of the tools, or parts of the microbalance with your fingers, or you could contaminate them with foreign materials. Make sure that all of the tools you will use for pilling have been previously rinsed with acetone and are clean before beginning. Using the balance brush, make sure that the balance is free from any debris. Label the lid of a 96-well plate with the sample set number. Always begin from weighing out the samples of a set before moving on to the standards. It is best practice to weigh the standards the day that you



will run them on the IRMS. The well plate should always be stored in a desiccator between pillings and prior to running the sample set on the IRMS.

Using forceps, begin by taking one 4×6 tin cup from the container and place it on the microbalance. Allow the balance to stabilize with the door closed before tarring. Next, open up the door and, using the small sample spoon, scoop a small amount of sample from the vial, making sure that you don't get any skin, bones, large chunks, or anything that doesn't look homogenized. Weigh out the appropriate amount of sample for the matrix you are working with. Close the balance door and let the weight stabilize before recording the weight of the sample on the loading sheet.

After opening the balance door up, use forceps to crimp the lip of the tin cup shut so that no sample is lost during folding. Then, using the flat-tipped forceps, fold the tin cup into a small tight cube on top of the stone surface. Make sure there are no sharp pieces sticking out of the cube and that the tin capsule is flat so that the cube does not get caught up on the autosampler. Using forceps, place the sample in the appropriate cell in the well plate and move on to the next sample. Make sure to wipe down all of the tools along with the stone surface with a paper towel between every sample, so as not to cross-contaminate samples. Repeat this process for all of the samples and standards in the set. Store the well plates in the desiccator until they are ready to be loaded on the IRMS.



When you are done using the tools, thoroughly rinse them in acetone and store them away until the next time they are used. Always place the lid on the tin that the capsules are stored in between uses. The balance should always be left with the door shut between uses. Turn off the balance at the end of the day.

### 8.3 Finalizing the Loading Sheet

Begin by inputting all of the weights of the samples and standards into the set's loading sheet. Double check that all weights were typed in correctly before moving on.

Click on the second tab of the template, labeled *Data*, and make sure that all of the information from the first tab has transferred over accurately. If you have removed rows, you might have to start at the first row and drag it down and then manually remove the rows that are not needed. You should be left with the same sequence number at the end of the data tab as on the set sheet.

Save this loading sheet to a USB so that you can access all of the required information upstairs when it is time to load the sample set. Make sure that you keep the hand-written loading sheet in the set's folder in case there is a discrepancy with sample weights.

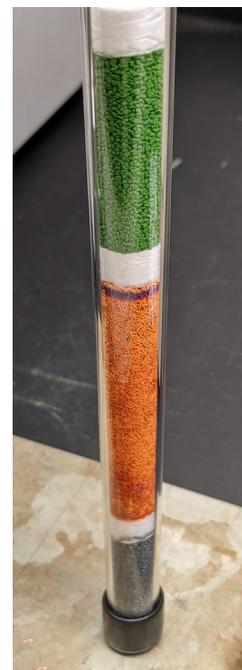
## 9.0 Loading the Samples on the Elemental Analyzer (EA)

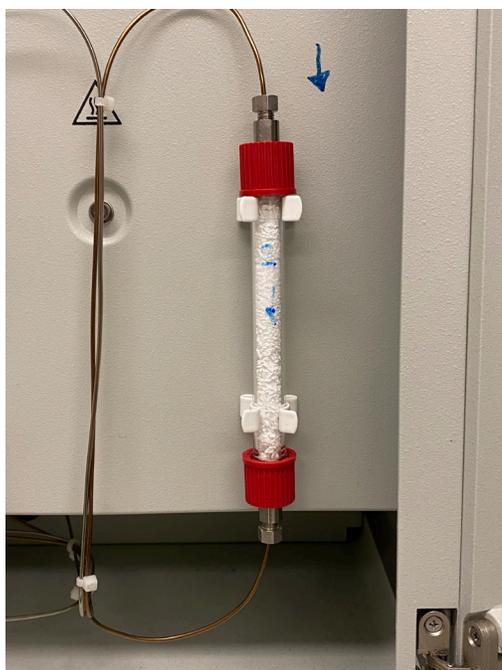
The day before you plan on running the set, check the instrument log book for maintenance needs and act accordingly. If the system requires a new reactor, allow the system to equilibrate and flush overnight before running any air/water tests or checking the background of the instrument.

### 9.1 Instrument Maintenance

The day before you plan to run a set on the IRMS, check the status of the instrument. Check how many samples have gone through the water trap, reactor, and ash tube. Replace the water trap after every 500 samples. Replace the reactor after 300 samples. Replace the ash tube after 124 samples and/or between sample sets.

Check the levels on all of the gas tanks along the wall, recording them on the clipboard hanging near the tanks. If any tank is below 500 psi, change it out with a new tank, and if the helium tank is low, use the switching valve to switch to the other tank that should be hooked up and open. This maintenance should be done the day before a run to allow the instrument to stabilize. You should also make sure that the correct instrument configuration has been selected in *Isodat Acquisition* in the bottom-left corner.





## 9.2 Running Linearity and On/Offs

On the morning of the day you are planning to run the sample set, on/offs and linearity checks need to be performed for both carbon and nitrogen. Make sure that the *ConFlo* and *EA* system set up has been selected in the bottom-left corner of *Isodat Acquisition*.

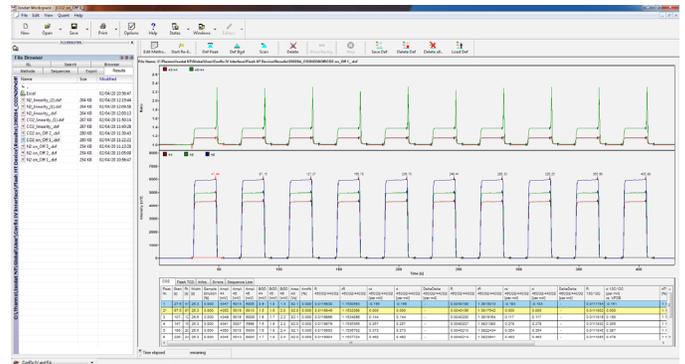
Begin by opening up *Isodat Acquisition*. Select the `ON_OFF's .seq` sequence from the sequences list in the bottom-left corner. Everything is all set to go once it has been properly loaded. Click the green **Start** button and select the **OK** button on the next screen. The sequence will continue through all of the on/offs and linearity checks. It takes about 2 hours to run the whole sequence.



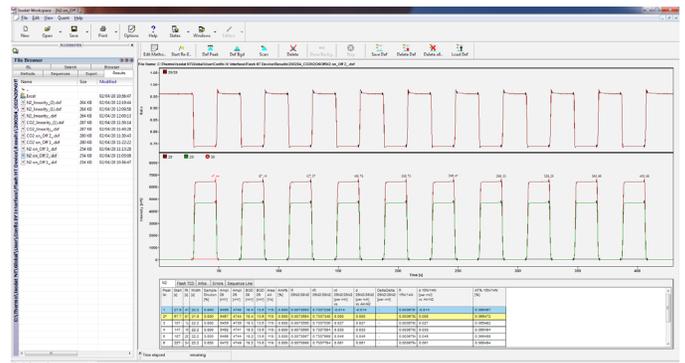
## 9.3 Checking the On/Offs and Linearity Checks

When the sequence has finished, open up *Isodat Workspace* and select the *Results* tab. Open up the on/off sequence that was run—it will have the date attached to the file name. Open each file by double-clicking. The standard deviations/slope should be checked and should be less than or equal to 0.06. If they are greater than 0.06, the on/offs should be run again; it may indicate that the system needs maintenance.

To calculate the CO<sub>2</sub> on/off, double click on the file titled CO<sub>2</sub> on\_off 4. Select the last one to run in sequence (it takes some time for the system to stabilize). When it opens to the screen, click on the column titled *d13C/12C* towards the bottom of the page—this should select the entire column. Right-click on the column and select **Calculate**. The standard deviation should be  $\leq 0.06$ .



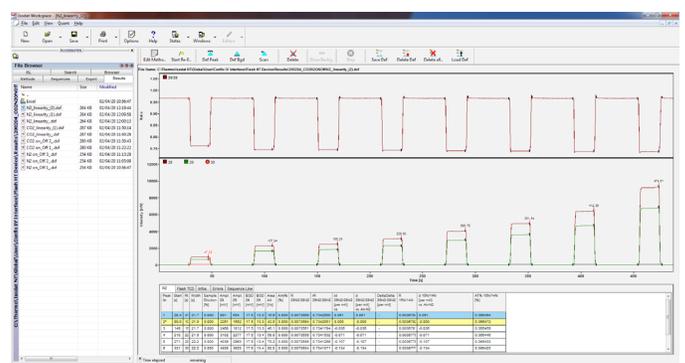
To calculate the N<sub>2</sub> on/off, double-click on the file titled N<sub>2</sub> on\_off 4. This is the last one to run in a sequence of three. When it opens to the screen, click on the column titled *d15N/14N* towards the bottom of the page—this should select the entire column. Right-click on the column and select **Calculate**. The standard deviation should be  $\leq 0.06$ .



To calculate the CO<sub>2</sub> linearity, double-click on the file titled CO<sub>2</sub>\_linearity\_(2). Minimize *Isodat Workspace* and open *Linearity Calculator* (an Excel file located on the desktop). Open *Isodat Workspace* back up and select the entire *Ampl. 44* column. Copy and paste it into the *Amplitude 44* column in *Linearity Calculator*. Next, select the entire *d13C/12C* column in *Isodat Workspace*. Copy-paste it into the *d13C/12C* column in *Linearity Calculator*. Excel will calculate the slope. The slope should be  $\leq 0.06$ .



To calculate the N<sub>2</sub> linearity, double click on the file titled N<sub>2</sub>\_linearity\_(2). Minimize *Isodat Workspace* and open *Linearity Calculator* (an Excel file located on the desktop). Open *Isodat Workspace* back up and select the entire *Ampl. 28* column. Copy and paste it into the *Amplitude 28* column in *Linearity Calculator*. Next, select the entire *d15N/14N* column in *Isodat Workspace*. Copy-paste it into the *d15N/14N* column in *Linearity Calculator*. Excel will calculate the slope. The slope should be  $\leq 0.06$ .



## 9.4 Checking the Background in the IRMS

After the diagnostic tests have been calculated, the IRMS background should be checked. Begin by opening up the *Background* template on the desktop. Log the date and follow the instructions in the template to record all of the necessary background measurements in the worksheet for that date. Make sure that you save when you are finished. You should also make sure that you have CO<sub>2</sub> selected as the reference gas and 44 in cup 2, 45 in cup 3, and 46 in cup 4 (if you right-click on cup 2 and enter 44 it will autofill the rest if CO<sub>2</sub> has been selected as the reference gas).

## 9.5 Building a Sequence in *Isodat*

To build a sequence, select **File** and then **New** and select **Sequence**. Type in the total number of samples and standards in the set and click **OK**. This will generate a blank sequence template. Plug the USB containing the sample set loading file into the computer. Open up the file and click on the *Data* tab. Begin copying the appropriate data from the *Data* tab and pasting it into the columns in the blank sequence template in *Isodat Acquisition*. The *Sample Name* column information is pasted into the *Identifier 1* column in *Isodat Acquisition*. The *Sample Type* column information is pasted into the *Identifier 2* column in *Isodat Acquisition*. Select the entire *EA Method* column, right-click, and select **Fill grid with Data**. Select the method `Flash Default\HTN_C_4sec_O2.eam`. Select the entire *Method* column, right-click, and select **Fill grid with Data**. Select the method `Methods\C_N\N_C_karl_jonelle_new.met`.

When all of the columns have been filled in, make sure that the *Peak Center* column has checks in all of the boxes for all of the runs. For the first blank, select **Start Blank Mean** for the *Type* column. For the second and third blanks, select **Add Blank Mean** for the *Type* column. All of the other samples and standards should have **Sample** as the *Type*. Right-click anywhere in the sequence and select **Fit Cells to Grid**.

When the sequence is completely filled out, go up to **File** and select **Save As**; name the sequence the name of the sample set. Next, go up to **File** and select **Print**. Place this printed copy of the sequence in the sample set folder.

Select the green **Start** button and begin to fill out the set's information. For the *Folder Name* select **Pre** and select **Date**. For the *File Name* select **Pre** and select **Identifier 1**. Deselect the **AutoEnum** option. Select **Excel** for the export and select **Modify Template**, add `08Dec2016testEAN_CO2.wke`, and select **Ok**. For the *Export File Name*, type in the set name. Finally, select **Interface Standby After Sequence** so that the instrument will automatically go into standby after the last sample has run. Before hitting **OK**, load the autosampler with the samples and manually advance the tray to drop the first pill into the chamber; see [Section 9.6](#).

## 9.6 Loading the EA Autosampler

Remove all of the trays from the top of the autosampler, making sure that the gear is set to zero (**0**). Remove the clear lid off of the first tray and begin loading the samples in the slots one by one until all of the samples have been loaded in the trays. Take care not to skip a slot, or to place two samples in one slot. After all of the samples have been loaded, stack the trays, starting with the **0–31** tray at the bottom and sequentially adding all of the trays on top of each other. Make sure to place the clear lid on top of the stack of trays before placing it on top of the autosampler. Ensure that the stack of trays is sitting evenly and level on top of the autosampler and manually advance the first tray, turning it clockwise one click to drop the first pill into the chamber. Make sure there is a click and that the tray has fully advanced to **1**.



## 9.7 Starting the Run

After the samples have been loaded and the tray has been advanced, press the **OK** button in *Isodat Acquisition* (where you left it in [Section 9.5](#)). Check to make sure that you have selected to put the instrument in standby after the set is done running. Also check to make sure there isn't a flag waving to indicate that the system is only going to run a selected number of samples out of the sequence. It is best to watch the first sample run to ensure that everything is running properly. Be sure to update the whiteboard by the computer, along with both the corresponding book and binder, with the sample information and number of samples through the reactor, water trap, and ash tube.

## 9.8 Post-Run

After the sequence is finished running, make sure that all of the samples have dropped from the autosampler by checking the trays and chamber for any that might have gotten caught or left behind. Reset the tray by manually rotating the gear back to zero (**0**) along with all of the trays. Check the gas tank levels and change out any tanks that might be low. Make sure that the system went into standby mode upon completion of the sequence by checking that the flows are turned down in the method.

If everything looks good, using the shortcut on the computer desktop called *EA Results*, find the Excel file generated for the set you just ran and save it on the USB you brought up with the loading template. Take the USB back down to your computer to process using *R*.

## 10.0 Excess Sample Archival

All unanalyzed field samples prepared for bulk stable isotope analysis can be stored at room temperature indefinitely. Samples are stored with tight caps in the stable isotope cabinet in Room 253E. Sample sets should stay together in a vial flat with the appropriate visible labeling for easy location. Sample set information should be written on the outside of each vial flat.

Samples will be archived for up to one year or a specified time period after the data have been validated, as agreed upon by the project leader and the principal investigator(s).

## 11.0 Processing the Data

Start by placing the Excel file generated by *Isodat* into the set's folder on the stable isotope server in the *Stable Isotope Projects* folder. The loading template and setup files should already be in the folder. Make a copy of all of the files in the tab *R Code*, and paste them into your folder.

### 11.1 Manipulating the Raw Data

Begin by opening up the `Manipulate raw data.R` file in the set's folder; it does not need to be saved. Fill out the set's name on line 35, replacing the *XXX*, and place the cursor anywhere above line 35. Hit the button **Run** at the top until a window opens up to select the raw Excel file that was generated by *Isodat*; double-click to select. Continue to hit the **Run** button until it goes through all of the data and you read a line that says *Saved*. Double-check that there are 4 peaks for each row. If there is an error message or there are less than 4 peaks for each row, troubleshoot accordingly. When everything looks good, you can exit out of the program without saving.

### 11.2 Processing the Data in R

Open up the *C/N Processing* folder and **Save As** the name of the set. Insert the set name on lines 8, 59, and 118. Answer the questions on lines 61, 62, and 63. Insert the run date on line 120. Insert the project name on line 128. Save the changes at the top of the page and hit **Knit**. Read through the PDF file that is generated to make sure that all QA pass. Copy-paste the `past_histidine28_plus_new_run.csv`, `past_SRM1946_plus_new_run.csv`, and `past_aspartic_plus_new_run.csv` files into the *R Code* folder, replacing the older versions. Place the folder in Jennie's inbox to be inputted into FileMaker.

## 12.0 Data

The laboratory will provide data tables and QA documentation suitable for QA assessment. All original data and data documentation developed by the laboratory for a given data package will be kept by the laboratory for at least one year after the data have been validated and reported; and, if requested, the data will be stored in the collection format for up to five years.

### 12.1 Data Review

All data are reviewed and evaluated by laboratory personnel. This review is undertaken by analysts who are responsible for ensuring that the analytical data are correct and complete, the appropriate SOPs have been followed, and that the QA results meet the acceptance criteria. It is the project manager's responsibility to ensure that all analyses performed by the laboratory are correct, complete, and meet project DQOs. The project leader has final review authority.

### 12.2 Data Storage

Processed data generated by *Isodat* are maintained in electronic files with frequent backup and storage onto an external hard drive. All final analytical results calculated in *R* are maintained in electronic database files on the stable isotope server maintained by IT, with frequent file backups and weekly backups onto tape for offsite storage. Bulk stable isotope results are also stored in the Environmental Chemistry Program's FileMaker database.

## 13.0 Quality Assurance

The Environmental Chemistry's bulk stable isotope established quality assurance provides guidelines for monitoring and documenting the quality of analyses so that a desired level of performance can be demonstrated and maintained. These guidelines are based on protocols established previously for specific projects under the National Oceanic and Atmospheric Administration (NOAA) and the Environmental Protection Agency (EPA), and have been adapted to new types of analyses and current technologies (Sloan et al. 2019).

### 13.1 Quantitation Range

For the EA/IRMS method, the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values can be affected if the mass spectrometer responses for the  $\text{CO}_2$  and  $\text{N}_2$  peaks are too small or too large. For field samples, results are reported if peak amplitudes for  $\text{N}_2$  (mass 28 and 29) and  $\text{CO}_2$  (mass 44 and 46) are between 500 and 12,000 mV. Samples that do not meet the above criteria are reanalyzed. If peak amplitudes are near their limits, the accuracy of the result is less certain. Sample results are footnoted to be used with caution if the peak amplitudes for  $\text{N}_2$  (mass 28 or 29) or  $\text{CO}_2$  (mass 44 or 46) are between 9,000 and 12,000 mV or between 500 and 750 mV. The corresponding delta and weight percentage (Wt%) measurements will not be reported if the sample's peak amplitude for mass 28 or mass 29 is >12,000 mV or if its peak amplitude for mass 44 or mass 46 is < 500 mV.

## 13.2 Instrument Calibration

The EA/IRMS method requires at least two delta levels of calibration standards (histidine and aspartic acid, prepared in-house) for analyte ratio measurements. The  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values for the calibration standards are assigned using primary standard materials from IAEA (IAEA CH-7) and the United States Geological Survey (USGS 40 and USGS 41a).  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  must be calculated using linear calibration and at least five replicate analyses of each calibration standard, including at least one of each at the beginning and end of the batch after extreme points (if any) are identified and excluded during the continuing calibration verification (Section 13.3). The  $\delta^{15}\text{N}$  results and  $\delta^{13}\text{C}$  results for the included replicate analyses of the calibration standards versus the respective assigned  $\delta$  values must have a correlation of  $r > 0.9900$ ; if this criterion is not met, then the samples in the batch must be reanalyzed.

Minimum Frequency: At least two of each calibration standard at the beginning and end of every batch, and between every 15 or fewer field samples.

## 13.3 Continuing Calibration Verification (CCV)

For the EA/IRMS method, the CCV standards'  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values are evaluated using the applicable four steps as follows:

1. The amplitudes of  $\text{N}_2$  (mass 28 and 29) peaks must be between 500 and 12,000 mV and the amplitudes of  $\text{CO}_2$  (mass 44 and 46) peaks must be between 500 and 12,000 mV; otherwise, that analysis of the standard is excluded from the data set and not further evaluated. At least one analysis of each standard must remain at the beginning and end of the batch.
2. The standard deviation of  $\delta$  values in the replicate analyses of each standard must be  $\leq 0.25$  per mil (‰) for  $\delta^{15}\text{N}$  and  $\leq 0.35$ ‰ for  $\delta^{13}\text{C}$ ; otherwise, extreme points will be identified and removed (see Step 3).
3. An extreme point is defined as the CCV standard with the greatest difference in  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  from the median of all replicate CCV standards. Extreme points are identified and excluded in a stepwise process until the standard deviations meet the criteria in Step 2.
4. No more than 20% of  $\delta^{15}\text{N}$  or  $\delta^{13}\text{C}$  values in the replicates of each CCV standard can be excluded due to extreme values. At least one analysis of each standard must remain at the beginning and end of the batch.
5. Minimum Frequency: At least two of each calibration standard at the beginning and end of every batch and between every 15 or fewer field samples (same standards as those used for instrument calibration).

## 13.4 Reference Materials

There is no tissue SRM certified for ratios of stable isotopes of carbon and nitrogen. However, National Institute of Standards and Technology (NIST) SRM 1946 (fish muscle tissue) is used as an IRM. The assigned reference values for  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$ , Wt%N, and Wt%C for SRM 1946 are the mean of repeated in-house analyses of this IRM for stable isotopes of carbon and nitrogen. The laboratory's performance for the EA/IRMS method is considered acceptable if a minimum of three samples of IRM per batch meet four criteria as follows:

1. For each IRM sample, the amplitudes of  $\text{N}_2$  (mass 28 and 29) and  $\text{CO}_2$  (mass 44 and 46) peaks must be between 500 and 12,000 mV; otherwise, that sample of the IRM is excluded from the data set and not further evaluated.
2. The mean of the  $\delta^{15}\text{N}$  values and the mean of the  $\delta^{13}\text{C}$  values must be within their respective control limits (Equations 7–10).
3. The standard deviation of the  $\delta^{15}\text{N}$  values must be  $\leq 0.3\text{‰}$  and the standard deviation of the  $\delta^{13}\text{C}$  values must be  $\leq 0.2\text{‰}$ .
4. The mean of the Wt%N values and the mean of the Wt%C values must be within their control limits (Equations 11 and 12).

If both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  meet the acceptance criteria but a Wt% does not, then the  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  are reported for all sample types in the batch but Wt% and C/N ratio are not.

Equations:

- $\delta^{15}\text{N}$  upper control limit = reference value +  $0.3\text{‰}$  (7)
- $\delta^{15}\text{N}$  lower control limit = reference value –  $0.3\text{‰}$  (8)
- $\delta^{13}\text{C}$  upper control limit = reference value +  $0.2\text{‰}$  (9)
- $\delta^{13}\text{C}$  lower control limit = reference value –  $0.2\text{‰}$  (10)
- Wt% upper control limit =  $(1.05 \times \text{Wt\% reference value})$  (11)
- Wt% lower control limit =  $(0.95 \times \text{Wt\% reference value})$  (12)

Minimum Frequency: Two run at the beginning and end of every batch and one run in the middle, with a minimum of three samples of IRM per batch meeting the acceptance criteria.

## 13.5 Method Blanks

For the EA/IRMS analyses, the method blanks are tin cups with no sample added, which are analyzed in the same manner as the environmental samples. These blanks are used to correct the  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  sample values for traces of nitrogen or carbon materials in the tin cups, and are not a measure of contamination that occurred during sample processing. The  $\text{N}_2$  mass 28 and  $\text{CO}_2$  mass 44 peak amplitudes for all of the method blanks must be  $<50$  mV, or the source of contamination must be determined and corrective action taken.

Minimum Frequency: Three at the beginning of every batch.

## 13.6 Sample Replicates

For EA/IRMS analyses, replicate samples of the IRM are analyzed between every 15 or fewer field samples, with a minimum of three per batch, to show the performance of the EA/IRMS system. Duplicate or triplicate field samples are suggested for approximately every 10 field samples, but are not used for QA. There are no acceptance criteria for replicate field samples because many explanations exist for widely varying values (e.g., problems with the sample processing or the homogeneity of the starting sample) that are often outside the control of the analytical laboratory. However, within-sample variability of the results for replicate field samples may be useful to the researcher.

## 13.7 Reported Results

For stable isotope ratios,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values are reported as ‰, where  $\delta^{13}\text{C}$  is the difference in ratio of carbon isotope  $^{13}\text{C}$  to carbon isotope  $^{12}\text{C}$  in a sample relative to that in the Pee Dee Belemnite standard, and  $\delta^{15}\text{N}$  is the difference in ratio of nitrogen isotope  $^{15}\text{N}$  to nitrogen isotope  $^{14}\text{N}$  in a sample relative to atmospheric nitrogen used as the standard (Equations 13 and 14).

Equations:

- $\delta^{13}\text{C} = \left\{ \left[ \left( \frac{^{13}\text{C}_{\text{sample}}}{^{12}\text{C}_{\text{sample}}} \right) / \left( \frac{^{13}\text{C}_{\text{standard}}}{^{12}\text{C}_{\text{standard}}} \right) \right] - 1 \right\}$  (13)

- $\delta^{15}\text{N} = \left\{ \left[ \left( \frac{^{15}\text{N}_{\text{sample}}}{^{14}\text{N}_{\text{sample}}} \right) / \left( \frac{^{15}\text{N}_{\text{standard}}}{^{14}\text{N}_{\text{standard}}} \right) \right] - 1 \right\}$  (14)

For stable isotope ratios, Wt%N, Wt%C, and C/N ratios are also reported.

## References

- Herman, D. P., D. G. Burrows, P. R. Wade, J. W. Durban, C. O. Matkin, R. G. LeDuc, L. G. Barrett-Lennard, and M. M. Krahn. 2005. Feeding ecology of eastern North Pacific killer whales *Orcinus orca* from fatty acid, stable isotope, and organochlorine analyses of blubber biopsies. *Marine Ecology Progress Series* 302:275–291.
- Sloan, C. A., B. Anulacion, K. A. Baugh, J. L. Bolton, D. Boyd, P. M. Chittaro, D. A. M. da Silva, J. B. Gates, B. L. Sanderson, K. Veggerby, and G. M. Ylitalo. 2019. Quality Assurance Plan for Analyses of Environmental Samples for Polycyclic Aromatic Hydrocarbons, Persistent Organic Pollutants, Dioctyl Sulfosuccinate, Estrogenic Compounds, Steroids, Hydroxylated Polycyclic Aromatic Hydrocarbons, Stable Isotope Ratios, and Lipid Classes. U.S. Department of Commerce, NOAA Technical Memorandum NMFS-NWFSC-147. <https://doi.org/10.25923/kf28-n618>

# Appendix A: Instructions for Submission of Samples for Stable Isotopes Analyses

## NWFSC Environmental Chemistry Program

1. Prior to filling out any paperwork, please discuss your project with Paul Chittaro, (206) 861-7617, and/or Jonelle Gates, (206) 302-2445.
2. For an idea of sample weights required for stable isotope analysis, refer to this table:

Type	Minimum Sample Weight for Bulk C/N	Minimum Sample Weight for CSIA
Whole Body Fish	1 g	3 g
Fish Muscle	1 g	3 g
Mussel/Shellfish	2 g	3 g
Marine Mammal skin/Blubber	0.5 g	1 g
Vegetation	2 g	3 g
Invertebrates	1 g	3 g

3. Obtain the Submission Form for Stable Isotopes Analyses<sup>1</sup> (see Appendix B) from the link provided, or via e-mail from Jonelle Gates (jonelle.gates@noaa.gov) or Jennie Bolton (Jennie.Bolton@noaa.gov). This information will be used to schedule the analyses, track the data and results, and anticipate problems. *Note:* Non-NOAA personnel may need to request permission to access the form.
4. Fill out information about your project and the general nature of your samples on the first page of the form (the *Project Information* tab).
  - **Investigator:** The person at NWFSC submitting the samples for analysis, or the contact person for an external agency.
  - **Project Name:** A concise (4–8 word) name for the project, including the sampling year, especially if the project is multi-year.
  - **Project Description:** A brief description of what the project is about (no more than a paragraph is needed).
  - **Charge Code:** The billing code, contract number, etc., for the work to be done.
  - **Referring Agency:** Either the Program/Division at NWFSC, or the name of the external agency/office.
  - **Date of Request:** Date on which the request for analysis is made, after the samples are all available to NWFSC personnel and any financial agreements are finalized.
  - **Date Data are Needed:** This date should be set by agreement of the investigator and NWFSC personnel, depending on priorities and workload. If you have a meeting where data will be presented, please include this information to help set the laboratory priorities. We will try to accommodate these requests as best we can.
  - **Analyses Requested:** Check the boxes for each measurement required.

<sup>1</sup> <https://drive.google.com/file/d/1Zan5tFQUOtoMvsyRkdfhtAcpkV2SRNUI/view>

- **Sample Prep. Required:** Check these boxes for any sample preparation that will not be done by your group. Normally, the group submitting samples will freeze-dry and pulverize solid samples according to our recommendations (NOAA Processed Report NMFS-NWFSC-PR-2020-04, *Standard Operating Procedures for Measuring Bulk Stable Isotope Values of Nitrogen and Carbon in Marine Biota by Isotope Ratio Mass Spectrometry (IRMS)*, will be provided), and weigh the sample pills along with an appropriate number of standard pills. Additional charges will be incurred to the investigator if NWFSC personnel are required to do this prep work. We advise groups submitting samples to freeze-dry and pulverize solid samples according to the provided SOP. If your group has no experience in pilling sample capsules for stable isotope analysis, Environmental Chemistry personnel can weigh the final sample and prepare the capsules for analysis (subject to agreement).
  - **General Description and Number of Samples:** Provide sample description (matrix) and number of samples to be analyzed.
  - **What Precision is Required (in per mil) for the Analyses:** If known, please indicate the precision for the analysis. A trophic level represents a difference of ~3‰ for  $\delta^{15}\text{N}$ . Differences down to 0.5‰ can be measured, but this requires a larger number of replicates to be analyzed.
  - **Expected %N Range:** If known, please provide the range of values expected. Samples with very high or very low values may need to be prepared or analyzed differently from other samples, so this information needs to be known in advance and discussed with NWFSC personnel.
  - **Expected %C Range** – if known, please provide the range of values expected. Samples with very high or very low values may need to be prepared or analyzed differently from other samples, so this information needs to be known in advance and discussed with NWFSC personnel.
  - **Anything Unusual About Samples:** List any properties of any of the samples that might be expected to cause problems with the analyses. For example, homogeneity becomes a problem with samples that have a high percentage of lipid. We advise lipid extraction or the use of liquid nitrogen in these cases before homogenization. Any samples expected to be less than 5% nitrogen should be noted. Problem samples should be discussed with Paul Chittaro or Jonelle Gates at the outset (prior to sample preparation).
  - **In What Format Would You Like the Final Data:** Check whether you would like hard copies with tables, an Excel worksheet, or both.
5. Fill out information about your samples on the *Sample Information* tab of the template. Note that of the following information, those marked \* are absolutely required; the others will appear on the data report you receive and will aid us in tracking and identifying your samples, and in providing the results in a more complete format. One hundred sample rows are given in this tab, but you may submit more samples if you have them. Make the sheet as long as you need.
- **Sample Category:\*** Select one of the following (please don't abbreviate): *vegetation, fish, invertebrate, marine mammal, filtrate*.
  - **Sample Type:\*** Describe each sample as follows: *Tissue – skin, Tissue – muscle, Tissue – whole body, Tissue – fat, Tissue – egg, Tissue – blubber, wood, leaf, berry, bark, periphyton, algae*. For samples from the animal kingdom, please use the format *Tissue (space) (dash) (space) type* as shown in the examples above. Contact Jennie if you have any questions.

- **Site:** Where the sample is from. Examples: *Snohomish Station 3, Prince William Sound*. If known, please include the latitude and longitude coordinates of where the samples were collected.
- **Species:** Please provide the Latin genus and species names if possible, or to lowest level of classification (genus, family, order). Examples: *Tsuga heterophylla, Thuja plicata, Oncorhynchus tshawytscha, Tricoptera*.
- **Common Name:** The common name of the species. Examples (from Latin names above): *western hemlock, western red cedar, Chinook salmon, caddis fly*.
- **Field/Animal ID:\*** Your field identifier/specimen number for this sample. Examples: *SN3-0095-02, 006, 145-SNOmmmp*.
- **Jar/Vial #:** If the sample container has some additional identifying information, enter that information here. This is a text field, so any short combination of letters and numbers (excluding special characters) is allowed. You could also use this field to designate a treatment group. Examples: *1 of 2, 4cc WX*.
- **Life Stage:** Examples: *adult, larvae, fry, juvenile, lactating, pregnant, spawning*.
- **Collection Date:** Date the samples were collected in the field, if you would like this information to be displayed on your report of results.
- **Sample Weight:\*** Weight of the sample pill for analysis, if you are pilling your own capsules.
- **Notes:** Note anything about the samples that may cause problems with the analyses (for example, individual samples with nitrogen content less than 5% could be noted as *low N*).
- **Sample Location/Contact:** Let us know whom to contact when we are ready to analyze your samples, so they can show us where the samples are located.
- **Cassette Identification:\*** Identify each cassette included in your submission. A researcher, group, and/or project name is a good idea, along with a number. Example: *Elwha River Nutrients 2010*.
- **Cassette position:\*** Provide the well number (A1, C3, etc.) for each sample in its cassette. Put the position in a single spreadsheet column (e.g., don't record the position with an *A* in one column and a *5* in another; record the position as *A5*). You may add columns as needed; for example, for *Reach* or *Transect*, and we will accommodate these in reporting to the extent possible.

**Important:** After completing the list of samples, check over the list to ensure that:

- 1) Empty pills or pills that have too little sample to effectively analyze (check with Jonelle or Paul if you have questions) are removed from the list.
- 2) The sample list is sorted first by *Cassette ID* and then by *Cassette position*.
- 3) There are no special characters (i.e., /, \*, ?, ' and ,). You can use an underscore ( \_ ) to separate descriptions, if needed. Special characters are difficult to check for when loading samples, and they will cause a runtime error in the instrument due to OS limitations.

We recommend that samples be submitted in order so that the loading of the SI autosampler goes smoothly. This will greatly reduce the chance of errors from samples being loaded in the wrong order, as samples are usually loaded exactly in the order presented. The autosampler can hold 124 samples/standards in one run. The submitted list of samples may be broken up into multiple sets run over several days; you will need to ensure that sufficient standard and SRM pills are also submitted.

6. If you have additional information that you would like to have displayed as part of your report of the stable isotope results, or if you need the data presented or provided in a particular format, please discuss your needs with the data manager, Jennie Bolton (Jennie.Bolton@noaa.gov, (206) 860-3359, Room 248E).
7. Once the Excel submission form has been filled out, email a copy to Jennie Bolton (Jennie.Bolton@noaa.gov) so that the samples can be scheduled for analysis, according to the priorities set in Step 4.
8. A sequence will be generated based on the information provided along with some basic pilling instructions for the sample/standard preparer, printed off, and handed to the preparer prior to pilling.
9. If the samples are from a material that has not been analyzed before by the Environmental Chemistry Program, you may be asked to prepare several test samples so that an optimal sample weight range for the material can be established.
10. **If collecting your samples requires filtration:**
  - Make sure to only use glass-fiber filters. **Do not use paper filters.**
  - Try to get as little of the glass-fiber filter into the sample as possible. Pieces of glass fiber can cause problems with the instrument during the analysis.
  - Salt from seawater can compromise the analysis. When collecting samples in seawater, try to get as little of the water as possible in with the sample before freeze-drying.

# Appendix B: Submission Forms for Stable Isotopes Analyses

## Project Information

Fill out the following information regarding your request. See the Instructions for more information.				
Investigator:				
Project Name:				
Project Description:				
Charge Code:				
Referring Agency:				
Date of Request:				
Date Data are Needed †:				Data Manager use only
Analyses Requested:	<input type="checkbox"/> %C <input type="checkbox"/> %N <input type="checkbox"/> C/N Ratio <input type="checkbox"/> δ13C <input type="checkbox"/> δ15N			Date in projects db:
Sample Prep Required:	<input type="checkbox"/> Extract Lipid <input type="checkbox"/> Freeze Dry <input type="checkbox"/> Pulverize			Date in whitesheet db:
Description and Number of Samples:				Edit: 9/13/2004 JLB
What Precision is Required (in per mil) for the Analyses?*				
Expected %N Range:				
Expected %C Range:				
Is %N Content Less Than 5% for Any Samples?				
Anything Unusual About the Samples:				
In What Format Would You Like the Final Data?	<input type="checkbox"/> Hard copy data tables <input type="checkbox"/> Excel worksheet <input type="checkbox"/> Both			
* A trophic level is a difference of ~ 3 per mil, but measurements down to 0.5 per mil can be made using a suitable number of replicate analyses.				
† "ASAP" is not a date - please see the submission instructions.				

## Sample Information

\* Indicates information that is required - please see submission instructions for how to format the entries.

	*	*				*	*	*			*		
	Sample Category	Sample Type	Site (include Lat./Long. if possible)	Species	Common Name	Field/Animal ID	Cassette ID	Vial/Well #	Life Stage	Collection Date	Sample Wt (mg)	Notes	Sample Location/Contact
1													
2													
3													
4													
5													
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9													
10													
11													
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50													



U.S. Secretary of Commerce  
Wilbur L. Ross, Jr.

Acting Under Secretary of Commerce  
for Oceans and Atmosphere  
Dr. Neil Jacobs

Assistant Administrator for Fisheries  
Chris Oliver

**February 2020**

[fisheries.noaa.gov](http://fisheries.noaa.gov)

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Fisheries Service  
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